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ORIGINAL ARTICLE

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Visualizing rhizosphere chemistry of legumes with mid-infrared synchrotron radiation

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Abstract A bright synchrotron light source operated by the Lawrence Berkeley National Laboratory served as an external source for infrared (IR) microscopy of plant root microcosms. Mid-IR light from synchrotrons is 2–3 orders of magnitude brighter than conventional sources, providing contrast based on the chemical information in the reflected signal at a spatial resolution near the diffraction-limit of 3–10 μm. In an experiment using plant root microcosms fitted with zinc selenide IR-transmissive windows (50 mm·× 20 mm × 1 mm), we describe chemical differences and similarities within the root zone of mung bean (*Vigna radiata* L.), grown with or without phosphorus, and revealed by reflectance spectromicroscopy. Comparative root and root-exudate profiles are described in sand/silt culture over the wavelength range of 2.5 to 16 μm (4,000 to 650 cm^{-1}) in the mid-IR, the spectral region most useful for the analytical identification of small organic molecules. Root epidermal tissue of plants grown with low phosphorus showed a greater lipid contribution and less lignin than nutrient-sufficient plants. In the zone 200 μm from the root axis, control plants were enriched with simple sugars and monomeric lignin precursors. In low-phosphorus plants, the rhizosphere possessed IR signatures from protein and sugars. Individual soil minerals could be easily discriminated from biological material. Synchrotron IR spectromicroscopy, therefore, complements existing root imaging techniques.

Keywords Infrared signature · Phosphorus · Rhizosphere · Root imaging · Synchrotron radiation · *Vigna* (rhizosphere)

Abbreviations ALS: Advanced Light Source · FTIR: Fourier transform infrared · SR: synchrotron radiation · ZnSe: zinc selenide

Introduction

The rhizosphere is an operational description for the region of intense biological and chemical reactivity near (< 2 mm) the surface of growing plant roots. Plant developmental biologists have sought for a long time a method for the repeated analysis of root properties and growth without recourse to excavation and disturbance of the soil. Mid-infrared spectroscopy (3–15 μm wavelength) measures the contribution of vibrational signatures from particular organic and inorganic functional groups (Harris and Bertolucci 1989; Stuart 1997). Infrared (IR) methods have a very long history of contribution to analytical chemistry. Unlike many types of microscopy, IR spectromicroscopy provides chemical analytical information on the composition of the root zone, and acts as an in situ contrast reagent for living tissues. By coupling synchrotron radiation (SR) sources and existing infrared (IR) microscope technology, we seek methods to observe intact plants: (i) actively mine the environment for nutrients, (ii) make heavy metals less toxic to themselves, (iii) defend themselves from predators and (iv) establish useful symbioses and associations with soil microbes. In light of this multichannel, multipurpose communication, techniques for the identification and characterization of root exudates are essential.

Visible SR was first observed at General Electric in 1947 (Elder et al. 1947). Originally judged a waste of

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energy, SR is electromagnetic radiation emitted by charged particles as they accelerate along highly relativistic orbits. Large electron-storage rings have been constructed into which high-energy electrons can be periodically injected. Once there, magnetic optical components constrain the electrons into closed, relativistic paths for several hours, while SR is continuously emitted from the ring. Some of the photon radiation from bending magnets leaves the ring through tangential ports (referred to as Beamlines), and passes to experimental stations located outside the ring.

Careful study of the properties of this radiation (Duncan and Williams 1983) demonstrates that it has three very useful characteristics: (i) a continuous spectrum from the far IR to the X-ray region, (ii) high brightness due to the high current and small emittances in the storage ring, and (iii) time-structure, with pulse repetition rates from 1 to 100 ns. Unlike a laser, which emits very intense radiation at one (or a few) wavelength, bending magnet SR is a continuum source. There are two benefits to plant researchers from such a light source. First, if suitable sample holders can be designed, the same sample may be studied at many different wavelengths in the same experimental facility, providing complementary information (Holman et al. 1999; Myneni et al. 1999). Second, and more importantly, polychromatic IR light sources allow a sample to be interrogated simultaneously at many wavelengths (interferometry), rather than tuning through a wavelength region of interest as with dispersive spectrometers. We take advantage of rapid Fourier transform infrared (FTIR) microscopy in this paper to provide a non-destructive spectral contrast technique for living root tissues and their immediate soil-water environment.

Point ii relates to the physical property of "brightness", for which a more precise discussion is warranted. As opposed to the photosynthetic photon flux density (μmol m^{-2} s^{-1}) unit of plant science, brightness refers to photon flux (unit area)$^{-1}$ (unit bandwidth)$^{-1}$ (solid angle)$^{-1}$. Bright SR sources produce intense, broadband light in a very narrow cone. Figure 1 is on a log-log brightness scale, and compares the Advanced Light Source (ALS) with a conventional thermal IR source found in an FTIR spectrometer or IR microscope. The thermal emission source is modeled as a 1,200 K black-body spectrum. With a difference in brightness over the mid-IR region of approx. 1,000 fold, it may be appreciated that one can manipulate the microscope optics to focus the much smaller "source" from the synchrotron to spot sizes impossible to obtain with reasonable signal-to-noise ratio from a thermal source. This allows spatial characterization with the synchrotron IR beam at essentially diffraction-limited resolution (McKinney et al. 1997; Jamin et al. 1998), an order-of-magnitude improvement in each linear dimension. The importance of this improved resolution for plant studies lies in the small size of individual eubacteria (1–5 μm), and the ability to discern gradients of root-derived compounds. As well, SR is pulsed, with discrete bundles of high-energy electrons spaced by nanoseconds within the storage ring (Martin and McKinney 1998; Carr 1999). Reaction kinetics of enzymes can be studied, assuming that the reaction can be repeatedly initiated in a pump/probe manner. Four synchrotrons in the US (and several more in Europe) currently provide such broadband, non-coherent light throughout the infrared: the Advanced Light Source (Lawrence Berkeley National Laboratory, Calif.), the National Synchrotron Light Source (Brookhaven National Laboratory, N.Y.), the Synchrotron Radiation Center (Stoughton, Wis.), and the Synchrotron Ultraviolet Radiation Facility (National Institute of Standards, Gaithersburg, Md.). All experiments in this paper were conducted with the ALS.

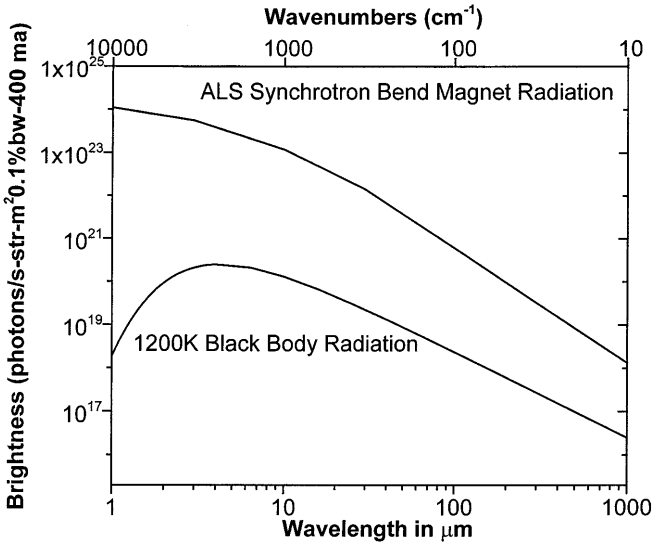

Fig. 1 Infrared brightness comparison of a 1,200 K black-body emitter and the ALS bending magnet at 400 mA of stored beam

Materials and methods

Plant root microcosms

Root boxes (dimensions 25 cm × 10 cm × 1.25 cm) were constructed of polycarbonate in two pieces, one solid backed with a drain slot, and a second thinner piece for the front of the root box having a machined slot for an IR-transmissive window. The window is a zinc selenide (ZnSe) trapezoid (Spectral Systems Inc; Hopewell Junction, N.Y., USA) of dimensions 50 mm × 20 mm × 1 mm thick. ZnSe is a chemically neutral, translucent, polycrystalline material with no significant IR absorption over 0.5–18 μm. However, the material does have a high index of refraction ($n = 2.4$), leading to reflection losses of approx. 17% per surface (Marion and Heald 1980). The window is held in place on each box with two Teflon washer-screw combinations. Prior to mounting, the window was carefully wrapped in aluminum foil with a small 0.5-mm-diameter hole cut into the foil. The foil-window assembly was then placed in a vacuum-deposition apparatus at the Scanning Electron Microscope facility in the Department of EPO Biology, University of Colorado, and gold deposited at low current for 50 s over the hole in the aluminum-foil mask. The gold was deposited on the root zone-facing side of the window, and serves as an

internal reflection standard for the IR beam. The two portions of the rhizobox were secured with large clips, covered with aluminum foil to suppress algal growth, and held upright at a slant in the greenhouse on flexible plastic test-tube racks. Each root box was constructed with a 40-mesh nylon drain-screen at the bottom.

Plant culture techniques

Horticultural sand (Rod McLellan Co.; San Mateo, Calif., USA) was acid-washed (1 M HCl) and vigorously rinsed with distilled water after sieving the soil to particle size > 53 μm. After air-drying, the sand was loosely packed into the upright root boxes, taking care not to unnecessarily scratch the ZnSe observation windows. Seeds of mung bean (*Vigna radiata* L.) were lightly pressed into the sand to a depth of 3 mm, and the microcosms watered and fertilized with 1/8th-strength Hoagland solution (Hoagland and Arnon 1950), either with or without phosphate as 1 mM KH_2PO_4. Micronutrients were modified for optimal growth of legumes (Fredeen et al. 1989), including the addition of both Co^{+2} and Ni^{+2} at 10 μM final concentration. Plants were fertilized once every other week, and watered, as needed, with 18-MΩ distilled water, until excess drained from each box. The root boxes received natural sunlight in a temperature-controlled greenhouse atop the Department of EPO Biology, University of Colorado from February until September of 1999. Depending on how the root boxes were oriented, it was usually quite easy to have the germinating seedling grow roots past the ZnSe window. At various intervals following germination, a subset of plants was carried by hand to the Lawrence Berkeley National Laboratory in California aboard a commercial jet for analysis.

Synchrotron IR microscope

The ALS at the Lawrence Berkeley National Laboratory is a third-generation synchrotron light source, and is operated as a National User Facility by the US Department of Energy. During most experiments described in this paper, the ALS operated with an electron energy of 1.9 GeV and a maximum beam current of 400 mA. The interval between beam refills was approx. 5 h. Beamline 1.4.3 at the ALS (McKinney et al. 1997; Martin and McKinney 1998) is dedicated to infrared spectromicroscopy, and collimated synchrotron light serves as an external input to a Nicolet Instruments (Madison, Wis., USA) Magna 760 FTIR spectrometer. The modulated light is then passed through a Nic-Plan IR microscope to perform either transmission or reflection microscopy. The sample stage of the microscope is controlled in the *x*-*y* plane, allowing automated spectral measurements across samples with steps as small as 1 μm. Due to the high IR absorbance of plastics, observations of the rooting zone were limited to the view afforded through the 50 mm × 20 mm ZnSe window. There are no common glass optical components (other than the visual eyepieces and a 10× sample-inspection objective) in the Nic-Plan IR microscope, which instead relies on all-reflecting Schwartzchild optics (Reffner et al. 1995). A 15× objective was used for all experiments, with the compensating ring adjusted for the approximate index of refraction of ZnSe. To minimize IR absorption by CO_2 and water vapor in ambient air, the optics were purged using dry air. Spectra for these experiments were collected in single-beam reflection mode with a wavelength resolution of 4 cm^{-1}, Happ-Genzel apodization, and 256 scans co-added for Fourier transform processing to produce one spectrum. In reflectance mode, the upper Cassegrainian objective serves a dual role as both the objective and condenser. Each resulting single-beam spectrum from the root box was ratioed to a gold reference spectrum recorded under the same conditions and acquired from the vapor-deposited spot in the upper corner of each ZnSe window. This normalization procedure removes the source-beamsplitter-detector efficiency functions, and minimizes IR spectral contributions from intervening water vapor and CO_2. Reflectance spectra or absorbance spectra were subsequently calculated. While Beamline 1.4.3 provides IR flux well into the far IR (< 100 cm^{-1}), the lower energy limit for spectra acquired through the IR microscope is 600 cm^{-1}, the cut-off point of its liquid N_2-cooled mercury-cadmium-telluride (MCT; Hg_{1-x} Cd_x Te) type-A detector. All spectra for this paper were acquired over the region from 4,000 to 650 cm^{-1} (2.5–16 μm).

Results

Mung beans grown in the rhizoboxes expressed large, branching root systems, some coinciding with the ZnSe observation window (tested up to 7–9 weeks following germination). Infrared reflectance spectra obtained of the root zone through the 15× objective of the IR microscope are shown in the figures as absorption spectra, and no re-scaling has been applied to them. Overall, IR reflection spectra demonstrated substantial differences among replicate mung bean plants exposed to either low-phosphorus or nutrient-sufficient conditions. For directly acquired IR spectral scans of the maturation zone root surface of nutrient-sufficient mung beans, we observed very strong absorbance features at 1,627, 1,399, and 1,269 cm^{-1}, with weaker ones at 1,003 and 775 cm^{-1} (Fig. 2A). Below the data appears the best spectral match obtained in comparisons, in this case from a wood-processing material known as 'sulfur-lignin'. By contrast, the similar developmental stage of the root surface in low-phosphorus supplied *Vigna* demonstrated weaker IR features overlapping those seen in the nutrient-sufficient roots, but stronger aliphatic contributions at 2,950–2,850 cm^{-1} and an isolated absorbance at 1,729 cm^{-1} (Fig. 2B). A comparison spectrum was assembled from a 2:1 mix of the best matches ('sulfur-lignin' and alkylated vegetable oils) to the original spectra.

When we turned our attention to the soil/water interface zone some 200 μm from the root axis of plants of both treatments, very distinct patterns emerged in the IR spectra. Spectral features below 2,000 cm^{-1} in the mid-infrared are considered to reside in the 'fingerprint region', and particularly noticeable are two strong IR-absorption families between 1,654 and 1,334 cm^{-1}, and 1,250 and 1,050 cm^{-1} (Fig. 3B) in the low-phosphorus-treated *Vigna*. The region from 2,000 to 650 cm^{-1} for low- phosphorus rhizosphere is shown in more detail in Fig. 4, including an IR noise comparison. Fewer in number yet strong features from 1,420 to 900 cm^{-1} are apparent in the rhizosphere of complete-nutrient *Vigna* (Fig. 3A). The solid substrate used for culturing the mung beans was acid-washed sand (> 53 μm particle size) with a mix of quartz, feldspar, olivine and hematite minerals (analysis not shown). When the IR microscope was focussed through a portion of the ZnSe window lacking visible roots below, one could obtain pure spectra of the growth medium. Silicate minerals in the rhizobox "soil" showed a prominent pair of Si-O IR features at 1,210–1,150 cm^{-1} and 1,050–1,040 cm^{-1}, and a smaller peak at 805–760 cm^{-1} (Fig. 5).

Visible in all raw spectra are water-vapor features from 4,000 to 3,500 cm^{-1} and 2,000–1,400 cm^{-1}, showing

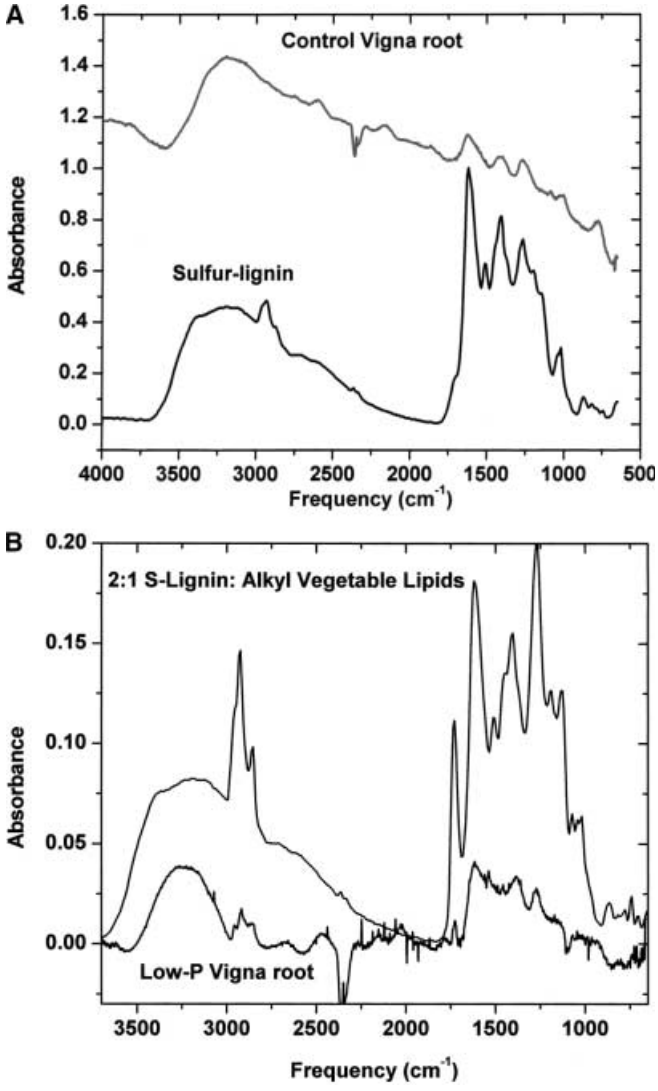

Fig. 2 IR reflectance spectrum from maturation zone of roots of nutrient-sufficient (**A**)and low-phosphorus (**B**) mung bean (*Vigna radiata*). The library spectrum of sulfur-lignin is shown for comparison in **A**, and a re-scaled library spectrum of 2:1 sulfur-lignin:alkylated C_{16}–C_{18} vegetable oils is shown for comparison in **B**

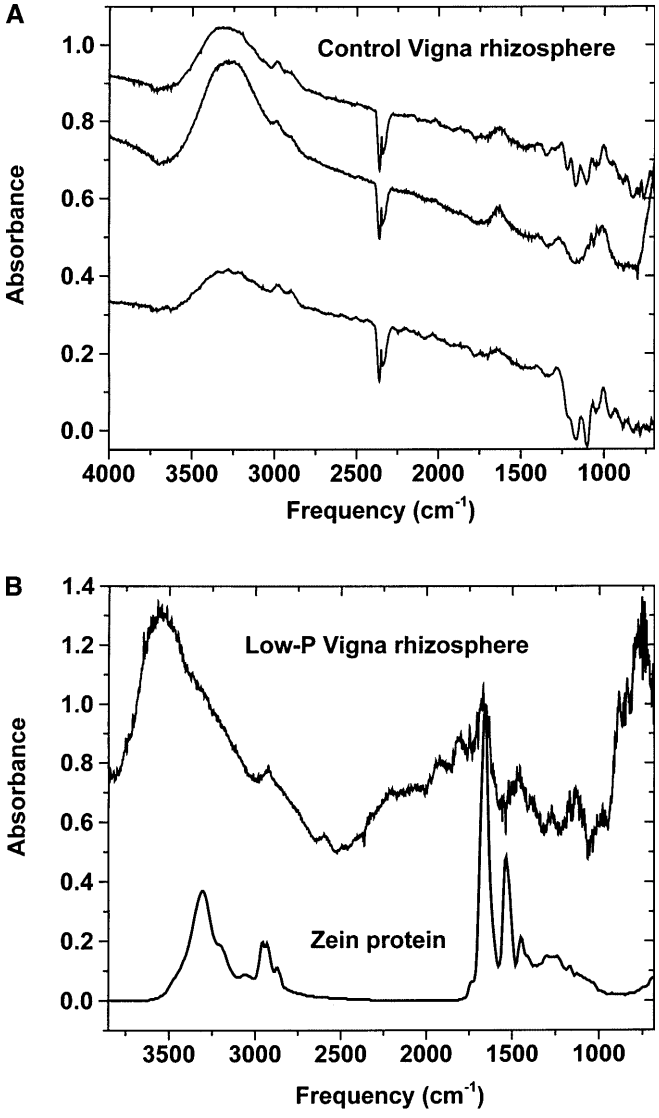

Fig. 3 IR reflectance spectra collected 200 μm from the root zone of mung bean seedlings with complete nutrients (**A**) or low phosphorus (**B**). The library spectrum of zein seed protein from corn is shown for comparison in **B**

the extensive rotational fine structure expected of compounds in the gas phase. This water-vapor contribution from the soil pore-space was subtracted from spectra to clarify underlying features. An IR doublet for CO_2 near 2,360 cm^{-1} is also common to root spectra due to respiration, but cannot be used for quantitative purposes after water-vapor subtraction (for example Fig. 2B).

The consistent aliphatic -CH_2 and -CH_3 stretch grouping in the range from 2,960 to 2,850 cm^{-1} arises from root lipid or cellulose absorptions, though additional IR features are required to differentiate lipids from carbohydrate polymers. Spectra acquired of the root epidermis clearly represent absorptions from C_{16} and C_{18} lipids in Fig. 2B, based on the presence of additional bands at 1,726 and 1,460 cm^{-1}.

Discussion

Infrared light reflected (4,000 to 650 cm^{-1}) through ZnSe windows in root microcosms of *Vigna radiata* differed in spectral signature depending on the level of phosphorus fertility to which plants were exposed. In the so-called 'fingerprint' region of the mid-IR (below 2,000 cm^{-1}), spectral differences almost certainly represent changes in the class of compounds surrounding the root zone. Our purpose in this study was to demonstrate that synchrotron IR spectromicroscopy could image particularly abundant compounds in the root zone. There is ample reason to suppose that such compounds influence overall soil chemistry and weathering processes (Banfield et al. 1999). The high brightness of the SR resulted in spectra from regions approximately 6–10 μm

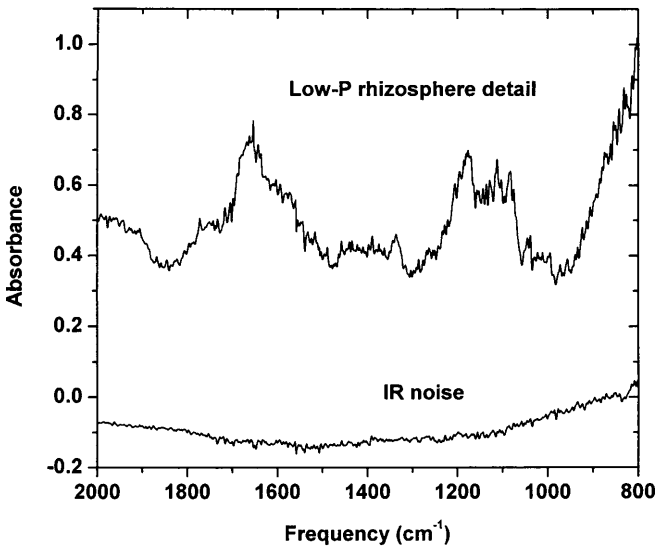

Fig. 4 Detail of IR spectra in the low-energy region for low-phosphorus rhizospheres, with a noise spectrum for comparison

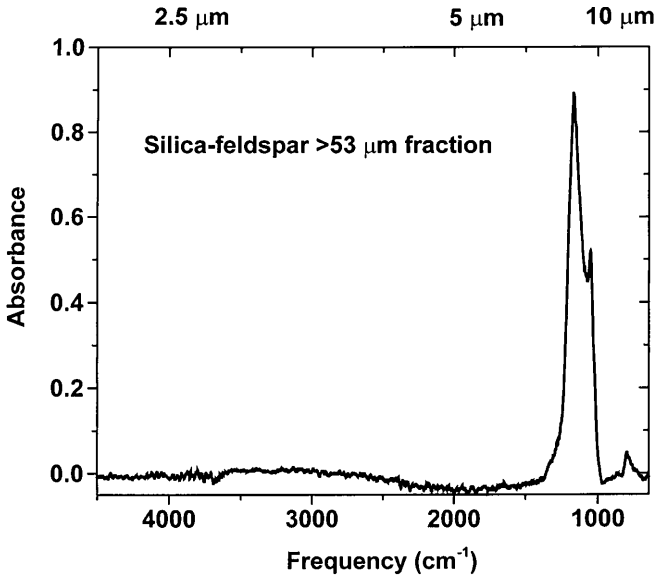

Fig. 5 Representative IR reflectance spectra from silicate minerals of the sand/silt growth substrate

square, and provides a way to dissect the rhizosphere into finer zones of chemical functionality.

The level of phosphorus supplied to *Vigna* appeared to alter the chemical signature of the outer epidermis of the root tissues in the maturation zone. Nutrient-sufficient plants had lower root/shoot ratios and demonstrated enrichment of lignin in the epidermis (Fig. 2A), whereas the low-phosphorus mung beans demonstrated membrane lipid dilution of the lignin features by comparison of the IR signatures (Fig. 2B).

In the soil-water interface zone surrounding the roots, we observed quite distinct chemical signals. Very low-energy (< 900 cm^{-1}) features observed in the IR spectra of control *Vigna* arise from simple sugars and/or di-, tri-, and tetrasubstituted benzene moieties (Fig. 3A). Derivatives of the plant phenylpropanoid pathway produce such compounds; the lignin precursor coumaryl alcohol is a 1,4-disubstituted ring, while coniferyl alcohol is a 1,2,4-trisubstituted ring. In their non-crosslinked forms, these lignin precursors should also give rise to a trans-ethylenic feature, visible in Fig. 3A at 960–970 cm^{-1}. The presence of monomeric lignin precursors in the root zone is a surprise and more detailed time-courses of their appearance and disappearance is required. In phosphorus-deficient microcosms (Figs. 3B, 4), the IR signal reflected from a region 200 μm distant from the root axis had spectral features arising from a complex mixture of proteins, aromatic carboxylic acids and sugars (Harris and Bertolucci 1989; Stuart 1997). The amide-I modes of proteins have a characteristic feature at approx. 1,650 cm^{-1}, with additional amide absorptions at 1,440 and 1,335 cm^{-1}. Primary alcohols possess IR features from 1,030 to 1,060 cm^{-1} (and at lower energies with unsaturation). Previous studies of legume responses to low-phosphorus document a general acidification of the rhizosphere (Marschner and Röhmeld 1983; Dinkelaker et al. 1989; Neumann et al. 1999), and increased exudation of aliphatic and aromatic carboxylic acids (Lipton et al. 1987; Hoffland et al. 1989; Ae et al. 1990). The identity of the protein(s) inhabiting the soil solution at high concentration is as yet unknown, but extracellular phosphatases have been observed in solution-culture experiments with tobacco (Goldstein 1992). The actual absorption envelope for the amide peaks is complex, and much can be deduced of the 2° structure of proteins from IR spectra (Stuart 1997). Clearly the microscopic technique will have to be combined with traditional biochemical assays to positively identify the function of the secreted protein(s).

With this synchrotron-based light source for IR microscopy, one can spatially resolve chemical processes in the rhizosphere to a finer scale than previously possible. State-of-the-art FTIR spectrometers possess internal thermal emission sources, an incandescent rod of silicon carbide at an equivalent black-body temperature of 1,200–2,000 K. Such a source, if used for IR microscopy, has an unapertured spot size of about 100 μm in each dimension. With a synchrotron IR source such as the ALS, the spot size is reduced to 3–10 μm (diffraction-limited performance in the mid-IR), a two-orders-of-magnitude improvement (Martin and McKinney 1998) in brightness over thermal sources. This is a key advantage of high-brightness sources. While it is true that one could use an aperture to reduce the spot size of a thermal source, there would also be an unacceptable loss of light as well. Given the extremely low reflectance of soils and biological materials in the mid-IR, the spectral features we have described from the roots of living plants and their soil environment would not be visible without the SR source.

High spatial resolution coupled with detailed molecular information cannot presently be obtained with any

other technology used in rhizosphere studies (Gregory and Hinsinger 1999). Thus, SRIR is complementary to soft X-ray SR microscopy of soil materials (Myneni et al. 1999), as well as ^{1}H-nuclear magnetic resonance (NMR) imaging of plants grown in suitably non-paramagnetic soils (Rogers and Bottomley 1987). Both direct multinuclear NMR (Fan et al. 1997) and chromatographic analysis (Ae et al. 1990) of isolated exudates provide very precise chemical assignments, though obtaining such chemical information from intact plant microcosms involves significant technical challenges, and does not preserve the spatial context of the exudates. Medical X-ray tomography, while useful for discriminating soil from living tissues, cannot provide spectral contrast beyond mean atomic number.

To be fair, we should also point out some limitations of this IR microscopy technique. First, the experiments described here depend on reflectance rather than transmittance measurements. The interaction of light with complex, organic-rich, soil pore water is complicated, and so many materials are inappropriate for use with IR spectromicroscopy. "Volume scattering" occurs when photons pass through several solid grains and incoherently scatter at every particle interface. When radiation of wavelength λ is incident on soil or biological particles of average size d, one can disregard volume scattering effects if $d > 10\ \lambda$ (Salisbury and Eastes 1985; Ramsey and Christensen 1998). If not, as the average particle size in a rhizobox decreases (i.e. towards the clay-rich fraction), the potential for reflected radiation to escape the surface and be detected by the IR microscope decreases. This has so far been confirmed by experiments in which we acquired spectra of lupins grown in high-organic-matter soils collected in alpine and grassland settings from Colorado (Raab et al. 1999), as well as fine-textured soils of a dry tropical forest in Hawaii. Little or no reflected signal could be detected in these soil microcosms, other than very strong lattice modes from silicate minerals. We thus chose a substrate of relatively uniform mineralogy and particle size of > 53 μm (i.e. the silt and sand fraction) to provide the maximum possible spectral contrast between the aqueous environment of the roots and the soil matrix (Fig. 5). Considering the Earth's major soil groups, perhaps only a handful will be well modeled by such microcosms. However, by germinating and growing plants directly in a sand/silt substrate, we hopefully provide roots with realistic mechanical and exudative properties compared to hydroponic- or agar-cultured plants. In a future paper we will consider the range of clay and organic matter contents that still provide useful spectra of root zone chemistry.

Secondly, we observe a complex mixture of compounds, and so interpretation of spectra must be automated to some extent. At Beamline 1.4.3, commercially available IR spectral libraries (nearly 16,000 spectra) are searchable with a common laboratory computer for initial, naive comparisons with plant/soil signatures, some of which are presented in the figures for comparison. One could deconvolve rhizosphere spectral mixtures if the reflected radiation from the soil (a heterogeneous solid bathed in an aqueous suspension of organic compounds) were a linear superposition of several end-member spectra proportional to their abundance in the soil solution. This "linear assumption" criterion for mid-IR spectroscopy is more easily satisfied than in the near-IR (Ramsey and Christensen 1998). Despite this proviso, near-IR spectroscopy finds many quantitative applications in agricultural and ecological studies. Selection of appropriate root microcosms and sampling schemes (Polonenko and Mayfield 1979; Faber et al. 1991) will extend quantitative measurements to mid-IR studies of plant and microbe interactions. In these initial experiments, IR spectra acquired directly from roots growing in the rhizobox are comparable to those collected from either thin sections of plant tissues (Wetzel and Reffner 1993), or purified biochemical preparations of root cell walls (Zeier and Schreiber 1999).

In conclusion, we have provided initial evidence that an IR microscope externally illuminated with bright SR allows acquisition of high-spatial-resolution IR spectra of compounds distributed within the rhizosphere (and the root surface) of living plants. By utilizing ZnSe windows in the root microcosms, we observed spectral features near the low-energy limit (600 cm^{-1}) of sensitive mercury-cadmium-telluride type-A detectors currently available in IR microscopes. It should be possible to combine SRIR with other existing microscopic or analytical techniques to better determine the role, mobility and production rates of root exudates.

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